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Oral L-Glutamine Therapy for Sickle Cell Anemia: I. Subjective Clinical Improvement and Favorable Change in Red Cell NAD Redox Potential

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Previously, we demonstrated that there is an increased utilization of glutamine by intact sickle red blood cells (RBC) in conjunction with nicotinamide adenine dinucleotide (NAD) metabolism in vitro. In this report, we describe the in vivo effect of L-glutamine supplementation on total NAD, nicotinamide adenine dinucleotide reduced (NADH), and NAD redox potential of sickle RBC. Seven adult sickle cell anemia patients participated in this study. The exclusion criteria were pregnancy, previous or current use of hydroxyurea, and transfusion within 3 months of initiation of the study. After proper consent, L-glutamine was started at a dose of 30 g/day administered orally. Fasting blood samples were drawn at baseline and after 4 weeks of therapy by routine phlebotomy for evaluation of RBC total NAD and NADH levels. We found significant changes in both the NADH level and NAD redox potential (ratio of NADH to NAD^+ + NADH). NAD redox potential increased from 47.2 ± 3.7% to 62.1 ± 11.8% ($P < 0.01$). The NADH level increased from 47.5 ± 6.3 to 72.1 ± 15.1 nmol/ml RBC ($P < 0.01$). The total NAD level demonstrated an upward trend (from 101.2 ± 16 to 116.4 ± 14.7 nmol/ml RBC) but this was not statistically significant. Our data show that oral L-glutamine can significantly increase the NAD redox potential and NADH level in sickle RBC. These changes may decrease oxidative susceptibility of sickle RBC and result in clinical benefit. Am. J. Hematol. 58:117–121, 1998.

Key words: sickle cell anemia; NAD; redox potential; L-glutamine

INTRODUCTION

Sickle cell anemia is one of the most prevalent hereditary disorders with prominent morbidity and mortality. The pathophysiology of this disease has been attributed to abnormal biophysical properties of aggregated hemoglobin S and other changes leading to hemolysis with moderate to severe anemia, decreased deformability of red blood cells, and increased endothelial adhesiveness resulting in compromised blood circulation. Recent studies have shown that oxidative phenomena play an important role in the pathophysiology of sickle cell anemia [1–8]. Previously, work from this laboratory has shown that sickle red blood cells (RBC) have a decrease in nicotinamide adenine dinucleotide (NAD) redox potential manifested by a lower ratio of nicotinamide adenine dinucleotide reduced (NADH) to total NAD (i.e., NADH plus NAD^+) and a compensatory increase in total NAD [9]. In addition, we have shown that there is a significant increase in the rate of transport for glutamine, a precursor for NAD [10]. Further work on glutamine in sickle RBC has also shown that there is decreased Km and increased Vm for glutamine transport in sickle RBC [10]. The intracellular level of glutamine on the other hand was not elevated; instead, the level of glutamate, a byproduct of glutamine in NAD synthesis, was increased in sickle RBC in comparison to high reticulocyte controls [10]. As the Km of NAD synthetase for glutamine has been known to be higher than the mean concentration of intracellular glutamine [11], we hypothesized, in conjunction with these data, that supplemental glutamine may increase the activity of NAD synthesis, thereby countering the oxidant-dependent pathophysiology of sickle

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RBC. Therefore, we conducted a pilot trial to examine the clinical and biochemical effects of oral L-glutamine supplementation in sickle cell anemia patients. We monitored hematologic parameters, NAD, NADH, and subjective clinical response. L-glutamine has been used widely as an oral supplement for various indications for decades with no evidence of major side effects[12,13].

MATERIALS AND METHODS

Materials

Spectrophotometric-grade ethanol was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Study Subjects and Blood Samples

After proper consent, seven adult sickle cell anemia patients, greater than 18 years of age (19 to 60 years old), with diagnosis of sickle cell anemia (homozygous Hb S) participated in the study. The exclusion criteria were (1) pregnancy, (2) transfusion of blood product in the previous 3 months, and (3) current or previous treatment with hydroxyurea. The patients were seen by the investigators at baseline, and after 4 weeks of therapy for interviews and collection of blood samples. In addition, during the study, each participant was seen by the investigator weekly or bi-weekly for brief clinical assessment and interview. Focused interviews were conducted on chronic pain, energy level, usage of narcotics, and activity levels throughout the study. Blood samples were obtained by routine venipuncture using heparin coated tubes (15 U heparin/ml whole blood) to prevent coagulation.

Administration of Glutamine

Ten grams of pure L-glutamine were administered three times a day orally to each participant for 4 weeks. This amino acid at the dose indicated has been shown to be safe for oral administration with essentially no adverse effects [14,15]. The powder was mixed with a glass of water, juice, or soft drink for administration. Compliance was monitored by frequent clinic and telephone encounters. The investigators contacted the participants at least once or more per week to interview regarding compliance and clinical status including adverse effects of the study medication.

Extraction of Reduced Pyridine Nucleotides

Extracts were prepared from whole blood immediately after phlebotomy. Twenty microliters of blood were mixed with 1,980 μl of a solution containing 10 mM nicotinamide, 20 mM $NaHCO_3$, and 100 mM Na_2CO_3 at 0°C. The mixture was frozen in a dry ice-acetone bath, thawed quickly in a room temperature water bath, and promptly chilled to 0°C. To destroy the oxidized form of NAD, 700 μl of this mixture as incubated at 60°C for 30 min. The mixture was then chilled to 0°C. Both the heat-treated extract that contained NADH and the unheated extract that contained both the reduced and oxidized forms of NAD were immediately analyzed using spectrophotometric cycling assays [16].

TABLE I. NADH, Total NAD, Redox Potential, and Hemoglobin at Baseline and After 4 Weeks of Glutamine Administration*

	Baseline (N = 7)	Week 4 (N = 7)	*P*
NADH (nmol/ml RBC)	47.5 ± 6.3 (41.2–57.0)	72.1 ± 15.1 (52.4–96.0)	<0.01
Total NAD (nmol/ml RBC)	101.2 ± 16.0 (77.7–118.0)	116.4 ± 14.7 (98.3–132.1)	n/s
Redox potential (%)	47.2 ± 3.7 (42.7–54.1)	62.1 ± 11.8 (48.4–80.7)	<0.01
Hemoglobin (g/dL)	8.5 ± 1.2 (7.1–10.6)	8.7 ± 1.2 (7.1–10.7)	n/s

*Values in parentheses indicate range. NADH, nicotinamide adenine dinucleotide reduced; NAD, nicotinamide adenine dinucleotide; RBC, red blood cells.

NADH and Total NAD Assays

NAD was assayed using spectrophotometric enzymatic cycling assays that measure both the oxidized and reduced forms of the nucleotide. This was a slight modification of the Bernofsky and Swan method [16]. The reaction mixture contained 100 μmol Tris-HCl, pH 8.0, 2 μmol N-ethyldibenzopyrazine ethyl sulfate (PES), 0.5 μmol 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 0.2 mg alcohol dehydrogenase (ADH), 600 μmol ethanol, and extract in a total volume of 1.00 ml. The reaction mixtures were pre-incubated for 5 min at 37°C and started with the addition of ethanol. Under these conditions, the rate of increase of absorbance at 570 nm was linear for at least 10 min and proportional to the quantity of pyridine nucleotide present. Blanks were used in the assay system to correct for background rates due to variable small quantities of tightly bound NAD in the ADH preparation.

Statistical Analysis

Paired *t*-test was used to evaluate the differences of values before and after the glutamine therapy for levels of NAD, NADH, NAD redox potential, and hemoglobin.

RESULTS

The participants took 30 g of L-glutamine orally each day for 4 weeks. After overnight fasting, blood samples were drawn for analysis of total NAD, NADH, and NAD redox potential. The summary of the results at baseline and at the end of 4 weeks is shown in Table I.

TABLE II. Subjective Clinical Response in Seven Hemoglobin SS Patients After 4 Weeks of L-Glutamine Therapy (30 g/day)

	Increased	Decreased	No change
Energy level	7	0	0
Activity level	6	0	1
Chronic pain level	0	7	0
Narcotics dosage	0	6	1
Adverse reactions: none			

There was a significant increase in NADH level leading to an increase in NAD redox potential (ratio of NADH to NAD^+ plus NADH, i.e., total NAD) after 4 weeks of L-glutamine administration. The total NADH level increased from 47.5 ± 6.3 to 72.1 ± 15.1 nmol/ml RBC ($P < 0.01$). Thus, the NAD redox potential increased from 47.2 ± 3.7 to 62.1 ± 11.8% ($P < 0.01$). In each participant, we observed consistent improvement in the redox potential to near normal to normal range (50–60%) or above [17]. There was one patient whose elevation of NAD redox potential increased only to 48.4% from 42.7%. In this patient, although the increase in the redox potential was relatively small, there was still a significant increase in NADH (from 40.9 to 60.7 nmol/ml). In this case, the increase in total NAD was relatively higher than in others (by increasing from 91.0 to 125.4 nmol/ml), resulting in a smaller increase in the redox potential, which is the ratio of NADH to total NAD. In terms of total NAD level, a trend upward could be demonstrated with an increase of the level from 101.2 ± 16.0 nmol/ml RBC at baseline to 116.4 ± 14.7 nmol/ml RBC at 4 weeks. This was a remarkable contrast compared to the significant increase observed in the level of NADH. There may be a mechanism leading to this disproportionate rise in NADH compared to total NAD, which is responsible for the elevation of the NAD redox potential.

Hemoglobin levels were measured in conjunction with NAD assays. In comparison to the baseline value, hemoglobin levels did not change significantly after 4 weeks of L-glutamine administration.

Clinically, within 4 weeks, all of the participants reported an improvement in overall energy level accompanied by increased activity level. We cannot attribute the above clinical changes to the effect of glutamine upon NAD metabolism in RBC at this point; nonetheless, the observed clinical effects were consistently positive. In addition, each patient also subjectively reported various degrees of decrease in chronic pain. In fact, six of the seven patients who participated in the 4-week study reported a decrease in daily narcotic usage. Although the clinical results appear consistent with the biochemical data, we must emphasize that these are subjective uncontrolled clinical data. No adverse effects of L-glutamine were reported by any of the patients (Table II).

DISCUSSION

Sickle cell anemia is one of the most devastating disorders affecting mostly people of African descent. The complications of this disease may start as early as in utero especially if the mother is also affected with the disease. Complications include miscarriage, fetal deformity, hand-foot syndrome, splenic sequestration crisis, cerebrovascular accident, infection, and vasoocclusive painful crises [18]. Over many years, different methods for managing this disease have been proposed and studied. As a result, over the last three decades there has been some overall improvement in the understanding and care of sickle cell anemia patients. However, this improvement has been mainly in supportive care such as early recognition and intervention of infection [19]. During recent years, we have seen some progress and developments in attempts to curtail the basic pathophysiology of sickle cell disease including hydroxyurea, bone marrow transplantation, and gene therapy. Unfortunately, there are major limitations with each of these modalities. Gene therapy is still under investigation at the pre-clinical level [20]. Bone marrow transplantation is available but limited only to those with a donor and is very costly [21]. It also has significant morbidity and mortality during the early phase of treatment [22]. Therefore, it is currently recommended only for very severe cases of sickle cell disease. Hydroxyurea therapy has resulted in definite improvement in sickle cell patients in terms of frequency of painful crisis but its long-term effect including possible leukemogenesis is not clear [23]. There are important questions yet to be answered regarding the safety of hydroxyurea especially in infants, children, and pregnant individuals [23–26]. In addition, hydroxyurea frequently causes neutropenia requiring close monitoring and frequent cessation of the medication [27]. An ideal agent would be one that is safe, effective, inexpensive, readily available, and easy to administer.

In this study, based on previous work from our laboratory, we have used orally administered L-glutamine to examine its effect on sickle RBC. L-glutamine is an amino acid that is readily available and has been shown to be safe for oral administration [12,13]. L-glutamine also is a precursor for NAD. We have previously shown that there is increased membrane transport and affinity for glutamine by sickle RBC [10]. Based on the data that the Km of NAD synthetase is higher than the intracellular concentration of glutamine in intact RBC, we hypothesized that glutamine supplementation may help promote synthesis of NAD. In vitro incubation of RBC with the precursors of NAD such as nicotinic acid has been shown to increase NAD in intact RBC [28].

The results were remarkable in our patients. With oral administration of L-glutamine at a dose of 30 g a day, there was a consistent and significant increase in red cell

NADH. In addition, we have seen an increase in the NAD redox potential. Although we were unable to observe a significant increase in the total NAD level, there was an upward trend. With a larger number of patients we may be able to demonstrate a significant increase in the total NAD level as well. In terms of increase in NAD redox potential, we can only speculate on its mechanism. What we observed was a disproportionate increase in NADH relative to total NAD. Some of the possible mechanisms leading to these changes include (1) direct action of L-glutamine as an antioxidant possibly through the glutathione pathway, (2) the effect of L-glutamine on 1,3-DPG, inorganic phosphate or reduced hemoglobin or other molecules involved in homeostasis of NAD and NADH levels, and (3) presence of an oxidative force peculiar to sickle RBC which remains constant; thus, as supplemental glutamine results in increased activity of NAD synthetase, the levels of total NAD and NADH increase and the effect of an oxidative force becomes proportionately smaller, which will in turn lead to an increase in the NADH level at an accelerated rate while the rate of increase for total NAD remains constant.

The increase in NAD redox potential may increase the defense against oxidative damage to sickle RBC. Although the clinical results are uncontrolled, all the participants reported a decrease in chronic pain by subjective assessment. In addition, six of the seven patients reported a decrease in the amount of narcotics required for their chronic pain. All the participants reported an increase in energy level while taking glutamine. In terms of adverse effects, there was no report of significant events. To attribute all of these positive subjective reports on the patient's clinical status to glutamine is premature. Nonetheless, it is notable that all of the participants reported an improvement in energy level and decrease in chronic pain. At this point, a controlled trial is necessary to evaluate the data obtained in this trial. However, if the data are confirmed in a controlled trial, a possible explanation for the favorable clinical outcome, aside from L-glutamine's interaction with the NAD redox potential, may be found in the neuronal interaction of the glutamate, because L-glutamine is an important precursor for glutamate, which is one of the major neurotransmitters [29–31].

In summary, we have observed an in vivo increase of NADH content and an increase in NAD redox potential in seven consecutive sickle cell anemia patients after daily oral supplementation of L-glutamine at a dose of 30 g a day. The patients tolerated the medication well without any report of side effects. There were subjective reports of improvement in their chronic pain level and energy level. Further study is currently in progress in an attempt to understand the effects of L-glutamine in sickle cell anemia patients at the physiological and clinical levels.

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